Research Protocol (v2, 20/1/2015)

STUDY TITLE

A PILOT RANDOMIZED CONTROLLED STUDY TO EVALUATE THE EFFECT OF INORGANIC NITRATE SUPPLEMENTATION DURING CALORIC RESTRICTION ON BODY COMPOSITION AND RESTING ENERGY EXPENDITURE IN OVERWEIGHT AND OBESE SUBJECTS.

STUDY INVESTIGATOR(S)

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1. INTRODUCTION

Inorganic nitrate supplementation is associated with improvements in vascular and muscular functions. The effects are mediated by an increased generation of nitric oxide which induces vasodilation of resistance vessels and enhanced P:O mitochondrial coupling. We hypothesise that inorganic nitrate supplementation amplify the effects of caloric restriction on metabolic efficiency and determine lowed losses of lean body mass as well as a lower decrease in resting energy expenditure during WL. We aim to conduct a randomized clinical trial to investigate the effects of inorganic nitrate on body composition and energy expenditure during weight loss in obese older subjects. The primary objective is to investigate whether inorganic nitrate + weight loss induces less mobilization of lean body mass compared to weight loss alone. The secondary outcomes include the evaluation of the effects of inorganic nitrate + weight loss on resting energy expenditure, resting blood pressure, hand grip strenght and micro-vascular function.
Thirty-six older (age range: 50-75y), obese (BMI: 25-40kg/m^2) overall healthy subjects will be randomized to two 2-week weight loss interventions. The first intervention will include WL + beetroot juice; the second intervention will include WL alone (negative control). Caloric restriction will be individualised to baseline energy requirements to induce a 40% caloric deficit. Subjects will attend the WL clinic every week. Measurements of body composition (BIA), muscular performance (hand grip strenght), vascular function (blood pressure, laser Doppler) and resting energy expenditure (Indirect Calorimetry) will be performed at baseline and at the end of the study (2 weeks). Saliva and urine samples will be collected at baseline and at each visit to monitor compliance. Blood samples will be collected at baseline for measurement of glucose, insulin, C reactive protein, total cholesterol, high density lipoproteins and triglycerides concentrations.

2. BACKGROUND

The world population is rapidly ageing. Between 2000 and 2050, the proportion of the world's population over 60 years of age will double from about 11% to 22% of the total

population. Ageing is associated with reciprocal changes in body composition (i.e., increase in fat mass and decrease in lean body mass) which are one of the factors leading to the increased cardiovascular and metabolic risk in older subjects. Currently, obesity in older aged subjects is on the rise but the evidence on the most effective and safe strategies for the treatment of obesity at this stage of life remains elusive. A major challenge is the loss of lean body mass during weight loss. Moderate weight loss in older obese subjects with diabetes and hypertension is still recommended as a first line of treatment, often prescribed with regular exercise sessions. The latter has been demonstrated to minimize muscle loss and therefore associated with greater improvements in cardio-metabolic functions and long term weight maintenance. However, physical mobility is often an issue in older obese subjects and therefore alternative strategies to reduce lean body mass loss are fundamental in obesity research.
Inorganic nitrate supplementation is linked to an increase generation of NO via a non-enzymatic pathway involving progressive reduction of nitrate into nitrite and NO. Nitric oxide has pleiotropic physiological functions including regulation of vascular tone and mitochondrial energy efficiency. All NOS isoforms (endothelial, neuronal, inducible) are expressed in skeletal muscles and it appears that NO plays a role in muscle mitochondrial biogenesis, glucose uptake, blood flow and muscle repair. The modulation of NO bioavailability can provide a therapeutic strategy to slow muscle loss during old age and prevents sarcopenia. Recent evidence has demonstrated that inorganic nitrate supplementation increases muscle energetic efficiency and performance during structured exercise in both young and older subjects.

3. AIM(S) OF STUDY

The aim of the study is to show whether inorganic nitrate supplementation during weight loss in overweight and obese subjects modify weight loss related changes in resting energy expenditure and body composition.

4. OBJECTIVES

The primary objective of the study is:

1. *Determine whether weight loss plus inorganic nitrate is associated with lower decrease in resting energy expenditure*

Secondary objectives include:

1. *Determine whether weight loss plus inorganic nitrate determines less loss in lean body in overweight and obese subjects*
2. *Test whether weight loss plus inorganic nitrate is associated with greater reduction in blood pressure and improvements in endothelial function*
3. *Investigate whether weight loss plus inorganic nitrate determine greater improvements in glucose, insulin and lipid levels*
4. *Investigate whether weight loss plus inorganic nitrate is linked to a greater improvement in physical and cognitive function.*

5. HYPOTHESIS

5b. Primary Hypotheses

The project is based on the hypothesis that inorganic nitrate will enhance the effects of caloric restriction on endothelial function and improve metabolic and vascular functions. This may be mediated by increased NO production, enhanced muscular mitochondrial function will improve muscle energetics and minimize the production of reactive oxygen species and local inflammation, which in turn will be linked to a decrease in loss of lean body mass following weight loss and reduce the expected reduction in resting energy expenditure. These benefits will be also impacting on other functions such as muscle strength and cognition function.

6. STUDY DESIGN

This is a two-arm, open-label, parallel randomized clinical trial. Subjects will be either randomized to either caloric restriction plus beetroot juice (as a rich source of inorganic nitrate) or caloric restriction alone (control).

7. STUDY SETTING/LOCATION

This is a single-centre study conducted at the Nutrition and Dietetics facilities of the Faculty of Medical School of the University Federico II of Naples, Italy.

8. STUDY POPULATION

Study will be conducted in overweight and obese middle-aged and older men and women attending the weight loss clinical facilities of the Nutrition and Dietetics facilities of the Faculty of Medical School of the University Federico II of Naples, Italy. Subjects will attend a first medical assessment visits and if eligible subjects will be invited to enroll the study after being thoroughly explained the aims, objectives and safety of the study by one of the investigators. Subjects will have at least one day to decide whether they wish to enroll in the trial. In total 36 subjects will be recruited (18 per arm).

9. ELIGIBILITY CRITERIA

<u>Inclusion criteria are:</u>
We aim to recruit 36 male and female older (50 - 75y) overweight and obese subjects (BMI Range: 25-40 kg/m^2) attending an nutrition clinic. Subjects will be non-smokers and weight stable.

Exclusion criteria are (reason for exclusion):
- Current participation in other research clinical studies
- Vegetarianism (likely to have very high nitrate intake)
- Weight change more than 3.0kg in the last 2 months (important influence on systemic metabolism and vascular function).
- Active cancer and any diagnosis of malignant cancer in the last 5 years (systemic effects on study outcomes).
- Diagnosis of chronic and acute metabolic and inflammatory conditions interfering with the study outcome (systemic effects on study outcomes). For example flu, Chrohn’s Disease, rheumatoid arthritis.

 Previous diagnosis of type 1 or type-2 diabetes treated with insulin (modification of regulation of intermediate metabolism). Type 2 diabetic patients treated with diet only or oral hypoglycaemic agents will be included in the study.
 Weight loss medications (sibutramine, orlistat, rimonabant) and history of bariatric surgery (weight loss related changes in systemic metabolism).
 Drugs: corticosteroids, sildenafil, aspirin, NSAIDs, diuretics, antacids, anticoagulants, nitrate-derived agents, anti-cholinergic, (all drugs may have either an effect on NO production or insulin sensitivity via different mechanisms).
 Subjects on hormonal therapies (oestrogens, thyroxine, progesteron), anti-hypertensive (Ca++ channel blockers, ACE inhibitors, beta-blockers,), statins and any other antidyslipidaemic agent, and psychiatric drugs (antidepressants, sedatives, antipsychotics) will be excluded if dose has been started/changed in the previous three months. (make sure that these disorders are under strict control to avoid interference with the study outcomes).
 Haematological disorders including self-reported anaemia, (risk for the participant and effects on the study outcomes).
 Major surgical operations interfering with the study outcomes (systemic effects on study outcomes).
 Alcohol intake >21 units/week for men and >14 unites/week women
 Blood donations in the previous 3 months.

10. STUDY OUTCOMES

10a. Primary Outcome

The primary outcome of the study is differences in resting energy expenditure and body composition between weight loss plus nitrate compared to weight loss alone after a 2-week weight loss treatment.

10b. Secondary Outcome(s)

Secondary outcome(s) include differences between the two arms in:

- Resting blood pressure
- Endothelial function
- Physical and cognitive functions
- Greater weight loss and lower drop-out rate at the end of the 2-week intervention

11. STUDY PROCEDURES

The study will be conducted according to the following procedures.

11a. Recruitment of participants

Subjects will be enrolled via the weight loss clinical facilities of the Nutrition and Dietetics facilities of the Faculty of Medical School of the University Federico II of Naples, Italy. Eligible subjects at the first medical assessment visit will be provided with information about the study asked whether they want to enroll into the trial.

11b. Randomisation

Participants will be randomised to one of the two interventions using a block-randomisation procedure. Blocks will consist of 5 participants per block and the randomisation sequence will be calculated using RandList for Windows.

11c. Study procedure

The study is essentially divided in three phases: screening/baseline, intervention and end of study. A summary of the three phases is described in Table 1 which also includes a breakdown by time of the various measurements taken at each phase.

Table 1: STUDY TIMELINE

Phases	Where	Details
Visit 1 (Medical Assessment)	Clinic (Morning)	Check eligibility criteria, signing informed consent, anthropometric measurements and blood pressure.
Agree to Participate and Eligible?		
No: Exclude from study		
Yes: Arrange appointment and **randomise** subject to intervention INTERVENTION 1: Weight loss plus Beetroot juice (1 per day) (18 participants) OR INTERVENTION 2: Weight loss alone (18 participants)		
Visit 2 (Dietary Planning and Baseline)	Clinic (Morning)	Saliva and urine sample Resting energy expenditure and endothelial function, BIA, hand-grip strength Cognitive function, dietary assessment and physical activity questionnaire Explain study intervention (beetroot juice plus diet or diet only). Provide beetroot juice for 2 weeks
Visit 3 (End visit after 14 days)	Clinic (Morning)	Saliva and urine sample Resting energy expenditure and endothelial function, BIA, hand-grip strength Cognitive function, dietary assessment and physical activity questionnaire

Medical Assessment (VISIT 1): Participants will arrive in the morning for their first medical assessment visit. The aim of the study will be explained to potential, eligible participants by the investigators and, if they agree to participate in the study, they will be invited to sign the informed consent. Anthropometric (weight, height) and BP measurements will be taken to

confirm eligibility. Subjects will be subsequently randomized to one of the three interventions (weight loss plus nitrate or weight loss alone) and they will be invited to return after one week for their baseline visit. Resting energy expenditure will be calculated using the Fredrix's equation and multiplied by 1.5 to obtain an estimate of total energy expenditure. A dietary plan will be then designed to obtain a caloric restriction of 40% of baseline estimated total energy requirements.

Dietary Planning and Baseline Measurements (VISIT 2): After 7 days, participants will return to the research centre to obtain information on the dietary and lifestyle intervention and perform the baseline measurements. Participants will arrive early in the morning in fasting conditions. They will provide a urine and saliva sample and then resting energy expenditure will be measured. This will be followed by measurement of endothelial function and resting blood pressure. Body composition will then be assessed by bioelectrical impedance and bilateral hand grip strength will also be assessed. Subjects will then complete a series of questionnaires including assessment of cognitive function, dietary assessment and physical activity level. After these measurements participants will be provided with details about the weight loss interventions and, if allocated to the beetroot group, they will be given 14 bottles of beetroot juice. Subjects will start the weight loss intervention the day after the visit and continue for 14 days. They will return to the clinic in the morning of the 15th day for their final visit and complete the end of the study measurements.

End Visit (VISIT 3): Subjects will arrive early in the morning the day after they had consumed their 14th beetroot juice bottle. They will be in fasting conditions and measurements will be repeated in the same order as at baseline.

Interventions: Each participant will be invited to start each intervention at the end of the baseline visit and continue for 14 days until they will return to weight loss clinic for their final visit (15 day). 18 participants will be allocated to the weight loss plus beetroot juice and 18 participants to weight loss alone. The two interventions are described below.

1) Weight loss: A hypocaloric low fat diet will be prescribed to each participant. The caloric restriction will be calculated as 40% of total energy requirements and macronutrients composition will be of approximately: CHO= 55-60%, FAT= 20-25%, PRO= 15-20%. The amount of high nitrate food (ie. lettuce, rocket, cabbage, spinach, broccoli, etc) will be similar between diets. Participants will be asked to drink the same water during the two weeks intervention. Participants will be asked to not change their habitual physical activity level and alcohol and caffeinated drinks consumption during the trial.

2) Inorganic Nitrate Supplementation: Participants will be asked to drink 70ml of concentrated beet root juice per day corresponding to an average supplementation of 300-400mg of inorganic nitrate per day. This amount of nitrate intake is commonly observed in subjects with a high intake of fruit and vegetables (particularly leafy vegetables) and it is significantly lower than the nitrate intake that may be observed in vegetarian subjects. There is no established health risk associated with either this level of nitrate intake or with beet root juice supplementation. However, at the screening visit participants will be asked whether they have an aversion for beet root or beet root juice and they will be excluded if they report problems with the consumption of these products. Subjects will be invited to drink 70ml of beetroot juice in the morning. They will be provided with a sheet to record the time of the consumption and if they will experience any problem. Participants will be considered not compliant to the intervention if they will miss and/or not complete two or more

supplementation days. Participants will be provided with the specific amount of beetroot juice to be consumed during the 14-day period.

Measurement Protocols

Questionnaires: Before and after the intervention participants will complete questionnaires on general health and lifestyle (IPAQ). In addition, participants' diet will be assessed using a standardized semi-quantitative food frequency questionnaire (FFQ). Subjects will also be administered a FFQ to evaluate nitrate intake. Cognitive tests (MMSE, TMT A and B) will also be administered at baseline and end of the study.

Body composition: Measurements will be performed at baseline and at the end of the 14-day interventions. Body weight, height and waist and upper-arm circumferences will be measured using standardised protocols.

Resting blood pressure: At baseline and end of the study resting blood pressure will be measured in triplicate using a manual BP monitor. Before the measurement participants will be invited to rest for at least 15 minutes. An appropriate cuff size will be utilised for the measurement of each subject which will be used for all other measurements of BP.

Endothelial Function: All the measurements will be undertaken in a temperature-controlled measurement room (~23 ± 1°C). Each subject will be invited to lie supine for a 5 min rest period. A laser Doppler device will be used to measure microcirculatory blood flow.

Hand-grip strength: Hand-grip strength will be assessed in both arms at baseline and after intervention to measure the maximum isometric strength of the hand and forearm muscles. A lightweight and portable dynamometer will be used. The researcher will follow a specific protocol for the measurement of hand-grip strength (i.e. allowing one practice trial, and then record the best of three attempts with 30 seconds rest between each of these).

Cognitive tests: All participants will complete a short battery of cognitive tasks that includes Mini Mental State Examination and the Trail Making Test [TMT Parts A and B].

Blood tests: Fasting blood tests will be performed by each participant before and after the intervention.

Urine and saliva collection: A spot urine sample will be collected in the morning (not first void) at the beginning and end of the intervention for the measurement of nitrate excretion. Stimulated saliva sample will be collected in 1.5ml eppendorf tubes. Samples will be stored at -20ºC until analyses

Dietary nitrate intake: It is essential to modify the diet to restrict nitrate intake in order to standardise the effects of the diet across the three different interventions as well as to increase the likelihood of detecting unconfounded effects of physical activity and nitrate supplementation on the main outcomes. Human exposure to nitrate occurs primarily through the diet because nitrate occurs in both plants and water. Nitrate is used as a preservative in cured meat but fresh meat contains little nitrate. Fruit and grains have a small impact on dietary intake of nitrate and the dietary advice offered will be recent reports on nitrate content of foods. Therefore diets will be standardized for the consumption: vegetables with high nitrate content (rocket, spinach, cabbage, beet root), tomato based products such as (tomato

sauces, ketchup, tomato juice), cured meat, cured seafood and cured fish, mature cheese. A detailed dietary plan will be given to each participant.

Compliance: Compliance with the dietary interventions will be assessed by monitoring the dietary intake at baseline, after 7 days (telephone interview) and at end of the study (14th day). Assessment of compliance with the physical activity will be assessed by asking subjects to complete a physical activity questionnaire.
Compliance with the nitrate (beetroot juice) supplementation will be evaluated by the completion of a daily questionnaire asking each volunteer about the time of the consumption of the juice as well as evaluating the changes in urinary nitrate concentration during the intervention (baseline, day 14).

12. STATISTICAL CONSIDERATIONS AND DATA ANALYSIS

12a. Sample size and statistical power

The sample size calculation was performed using G-Power (version 3). The model selected for the study was a t test model for independent measures. The outcome for the calculation of sample size was difference in REE between intervention and control. A difference between intervention and control of 150kcal/day (SD: ±150kcal/day). Power and significance levels were set at 0.80 and 0.05, respectively. Using these parameters we estimated a total sample size of 18 participants per group, i.e, 36 participants in total.

12b. Statistical methods

Normality of distribution of the variables and appropriate transformations (LogX, 1/X, X^n) will be performed if necessary. Summary data will be expressed as mean ± s.d. General linear models for repeated measures will be used to detect significant differences between the two intervention groups with and without adjustment for baseline levels. An interaction term (time x group) will be built to assess between-group differences in changes in the measured outcomes at the end of each intervention. Correlation analyses will be used at baseline to examine the association between outcome measures.

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